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FUNGAL AND PARASITIC INFECTIONS

Comparative Analysis of the Capacity of the *Candida* Species To Elicit Vaginal Immunopathology

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ABSTRACT The human fungal pathogen *Candida albicans* is the major etiological agent of vulvovaginal candidiasis (VVC). Despite this fact, other non-*albicans Candida* (NAC) species have frequently been reported, as well. Despite their presence in the vaginal environment, little is known about their capacities to elicit immune responses classically associated with *C. albicans*-mediated immunopathology, including neutrophil recruitment and proinflammatory cytokine signaling. Therefore, using a combination of *in vitro* and *in vivo* approaches, we undertook a comparative analysis to determine whether a representative panel of NAC species could colonize, induce immunopathological markers, or cause damage at the vaginal mucosa. Using a murine model of VVC, *C. albicans* was found to induce robust immunopathology (neutrophils and interleukin 1β [IL-1β]) and elicit mucosal damage. However, all the NAC species tested (including *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *C. auris*) induced significantly less damage and neutrophil recruitment than *C. albicans*, despite achieving similar early colonization levels. These results largely correlated with a notable lack of ability by the NAC species (including *C. dubliniensis* and *C. tropicalis*) to form hyphae both *in vitro* and *in vivo*. Furthermore, both *C. dubliniensis* and *C. tropicalis* induced significantly less expression of the *ECE1* gene encoding candidalysin, a key fungal virulence determinant driving VVC immunopathology. In order to determine the relative capacities of these species to elicit inflammasome-dependent IL-1β release, both wild-type and NLRP3$^{-/-}$ THP-1 cells were challenged *in vitro*. While most species tested elicited only modest amounts of IL-1β, challenge with *C. albicans* led to significantly elevated levels that were largely NLRP3 dependent. Collectively, our findings demonstrate that although NAC species are increasingly reported as causative agents of VVC, *C. albicans* appears to be exceedingly vaginopathogenic, exhibiting robust immunopathology, hypha formation, and candidalysin expression. Thus, this study provides mechanistic insight into why *C. albicans* is overwhelmingly the major pathogen reported during VVC.

KEYWORDS *Candida*, NAC species, VVC, immunopathogenesis, inflammasome, vaginitis, vulvovaginal

Vulvovaginal candidiasis (VVC) is a common mucosal infection in immunocompetent women overwhelmingly caused by the opportunistic fungus *Candida albicans* (1, 2). Symptomatic infection typically results in itching, burning, pain, and redness of the vaginal mucosa, often accompanied by vaginal discharge (3). VVC is the most prevalent human candidal infection, affecting ~75% of the female population at least once in their lifetime. Moreover, 5% to 8% of all women suffer from recurrent infections (RVVC), defined as >3 episodes per year, often necessitating continuous antifungal therapy (4, 5).

A landmark live-challenge study conducted by Fidel and colleagues described VVC as an immunopathology in which the host innate immune response is dominated by an

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influx of polymorphonuclear leukocytes (PMNs), is nonprotective, and ultimately drives the above-mentioned vaginal symptoms (6). Recently, it was demonstrated that the NLRP3 inflammasome, a cytoplasmic protein complex involved in the release of interleukin 1β (IL-1β) and IL-18, plays an important role in recruiting PMNs to the vaginal mucosa during *C. albicans* challenge (7, 8). Work from our laboratory has also established that fungal colonization of the vaginal mucosa alone is insufficient to drive PMN recruitment and proinflammatory cytokine signaling (including IL-1β), as hypha-deficient mutants exhibit robust asymptomatic colonization (9). Instead, we have recently shown that both hypha formation and downstream expression of *ECE1*, encoding the hypha-expressed toxin candidalysin, are the crucial virulence determinants driving neutrophil recruitment and damage at the vaginal mucosa (9, 10).

While the majority (~90%) of VVC cases are due to infection with *C. albicans* (1), other, non-*albicans Candida* (NAC) species have been implicated as causative agents of VVC, and their incidence is disproportionately high in certain geographic regions (11). Retrospective reports indicate that identification of NAC species during VVC episodes range anywhere from 5% to 50% of total *Candida* species isolated (12–25). Of these, *C. glabrata* is most frequently reported as the second leading causative agent (~5%), and the remainder are most commonly composed of *C. krusei*, *C. parapsilosis*, and *C. tropicalis* (11, 20, 23). Reported vaginal symptoms caused by NAC species are often milder than those experienced during infection with *C. albicans* (26). However, antifungal resistance and recurrence rates are typically higher with the NAC species, necessitating prolonged and/or alternative treatment options (23, 27). Even more troubling are several reports indicating that NAC species are being increasingly identified as causative agents of VVC (28, 29). Moreover, while the virulence mechanisms driving *C. albicans* immunopathogenesis at the murine vaginal mucosa are well described (e.g., hypha formation, candidalysin expression, and inflammasome activation), those responsible for immunopathology caused by NAC species remain poorly defined. Since routine surveillance of NAC species in VVC is still fairly uncommon (with the exception of *C. glabrata*), little is known regarding the true capacities of the NAC species to elicit immunopathology at the vaginal mucosa. This is further complicated by divergent clinical views on the role of NAC species during VVC: one school of thought accredits the NAC species with a true pathogenic function, while another suggests only an incidental bystander role during infection (11, 14, 30). Thus, clarifying which species of *Candida* exhibit vaginopathogenicity may allow physicians to make appropriate therapeutic decisions for treatment and simultaneously impede development of azole resistance in susceptible species. Therefore, to better delineate the pathogenesis of NAC species (including *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *C. auris*) at the vaginal mucosa, a comparative study of their capacities to elicit neutrophil recruitment, damage, and NLRP3 inflammasome-dependent IL-1β was undertaken.

RESULTS

Relative immunopathogenicities of the NAC species during VVC. In order to determine which *Candida* species are capable of colonizing and eliciting neutrophil recruitment, inflammatory cytokine production, and damage at the vaginal mucosa *in vivo*, a comparative analysis of a panel of reference isolates (i.e., those whose genomes have been sequenced) of both *C. albicans* and NAC species was undertaken. Using the murine model of vaginitis, we assessed these biomarkers of infection at both day 3 and day 7 postinoculation (p.i.) to determine potential differences between early and late responses. Surprisingly, colonization rates were similar for all the species tested at the early time point (Fig. 1A). However, colonization with strains capable of forming true hyphae (*C. albicans*, *C. dubliniensis*, and *C. tropicalis*) was generally higher and more consistent at day 7 (Fig. 1B). Despite this trend, colonization rates among species failed to reach statistical significance. *C. albicans* exhibited the highest level of vaginopathogenicity at both time points. The only other species that approached eliciting consistently high levels of PMNs and IL-1β was *C. dubliniensis*, the species most closely related

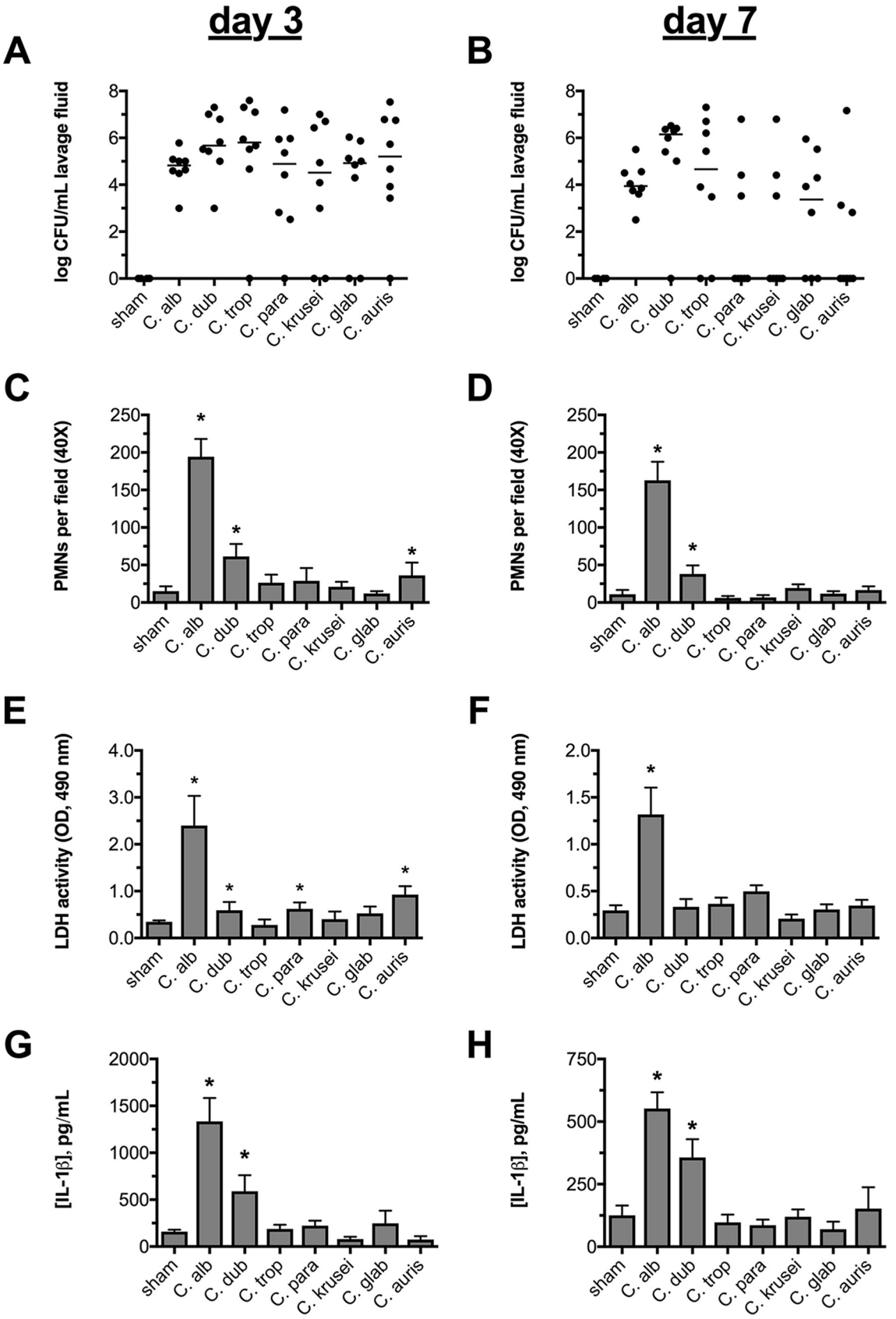

FIG 1 NAC species fail to elicit robust inflammation or mucosal damage at the vaginal mucosa. Groups of estrogen-treated C57BL/6 mice ($n = 8$) were intravaginally challenged with PBS, *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, or *C.*

(Continued on next page)

to *C. albicans*. Conversely, *C. tropicalis* (the only other reported true hypha former) was among the least immunopathogenic species tested. The remaining NAC species, including those that are reported to grow as pseudohyphal or yeast forms (e.g., *C. parapsilosis* and *C. glabrata*), elicited only marginal levels of inflammatory effectors that were not significantly elevated over those of sham-treated controls (Fig. 1C to H). However, the role of the yeast-hypha switch in driving VVC immunopathology could not be completely determined without comparing fungal morphology within the vagina.

Morphogenesis among the NAC species. In order to determine whether the lack of robust immunopathology observed among the NAC species was due to a failure in the ability to switch from yeast to hyphae, vaginal lavage fluids were stained to elucidate both neutrophils and fungi by microscopy. Intravaginal challenge with *C. albicans* led to robust levels of PMN recruitment (Fig. 2A, yellow arrows) and copious amounts of hyphal filaments (green arrows), indicating robust activation of the morphogenetic switch. In fact, *C. albicans* is found almost exclusively in the hyphal form during murine VVC. Analysis of lavage fluid from mice challenged with *C. dubliniensis* revealed only very sporadic filamentation and recruitment of relatively few PMNs (Fig. 2B). Interestingly, challenge with *C. tropicalis* revealed colonization with only yeast cells, as no hyphae or pseudohyphae could be detected throughout all the prepared slides (Fig. 2C). Unsurprisingly, this correlated with the presence of very few PMNs. Similarly, despite the capacity to form pseudohyphae, challenge with *C. parapsilosis* and *C. krusei* led to only the observation of yeast cells with few PMNs present (Fig. 2D and E). As expected, *C. glabrata* and *C. auris* (species restricted to the yeast morphology) were found in the vaginal fluid as only yeast forms among a few PMNs (Fig. 2F and G). Thus, these data are well correlated with previously observed results (Fig. 1), strongly suggesting that the capacity to form hyphae *in vivo* robustly drives the immunopathogenesis of VVC (9).

We next wanted to determine whether these morphogenetic phenotypes were restricted to colonization of the murine vagina or were more broadly conserved *in vitro* under hypha-inducing conditions. Planktonic growth of the isolates in RPMI medium (a tissue culture medium that robustly induces filamentation in *C. albicans*) led to very similar results, where *C. albicans* robustly formed hyphal filaments while *C. dubliniensis* and *C. tropicalis* formed only infrequent and comparatively stunted hyphae (see Fig. S1 in the supplemental material). The remaining species grew exclusively as yeast forms under these conditions. Therefore, hyphal growth observed *in vivo* at the vaginal mucosa is largely reflective of growth *in vitro* under conditions that permit filamentation, suggesting that the pathogenesis of each species/strain is under unique morphogenetic control. Moreover, these results also demonstrate that, among the strains tested, *C. albicans* exhibits the greatest pathogenicity (e.g., hyphal growth and mucosal damage) at the vaginal mucosa, likely explaining its dominance as the major etiological agent of VVC.

NAC species do not effectively elicit NLRP3 inflammasome-dependent IL-1β release. Since we and others have previously demonstrated an important role for the NLRP3 inflammasome in governing early neutrophil recruitment and inflammatory cytokine signaling (including IL-1β) during VVC, we wished to determine the relative capacities of the *Candida* species to stimulate NLRP3-dependent IL-1β release (7, 8, 31). In order to assess comparative pathogenicity, we challenged both wild-type (WT) and NLRP3$^{-/-}$ differentiated THP-1-derived human macrophages with equivalent amounts

FIG 1 Legend (Continued)
auris. Vaginal lavage fluid was assessed longitudinally at day 3 and day 7 for fungal burden (the horizontal lines indicate medians) by microbiological plating (A and B), for PMNs (means plus SEM) by microscopy (C and D), for the damage biomarker LDH (means plus SEM) by enzymatic assay (E and F), or for IL-1β (means plus SEM) by ELISA (G and H). Experiments in all the inoculation groups were performed in duplicate, and the data were combined. The data were tested for normality using the Shapiro-Wilks test. Statistical significance was calculated using one-way ANOVA and the Kruskal-Wallis posttest (nonnormally distributed data) or Dunnett's posttest (normally distributed data). *, $P < 0.05$.

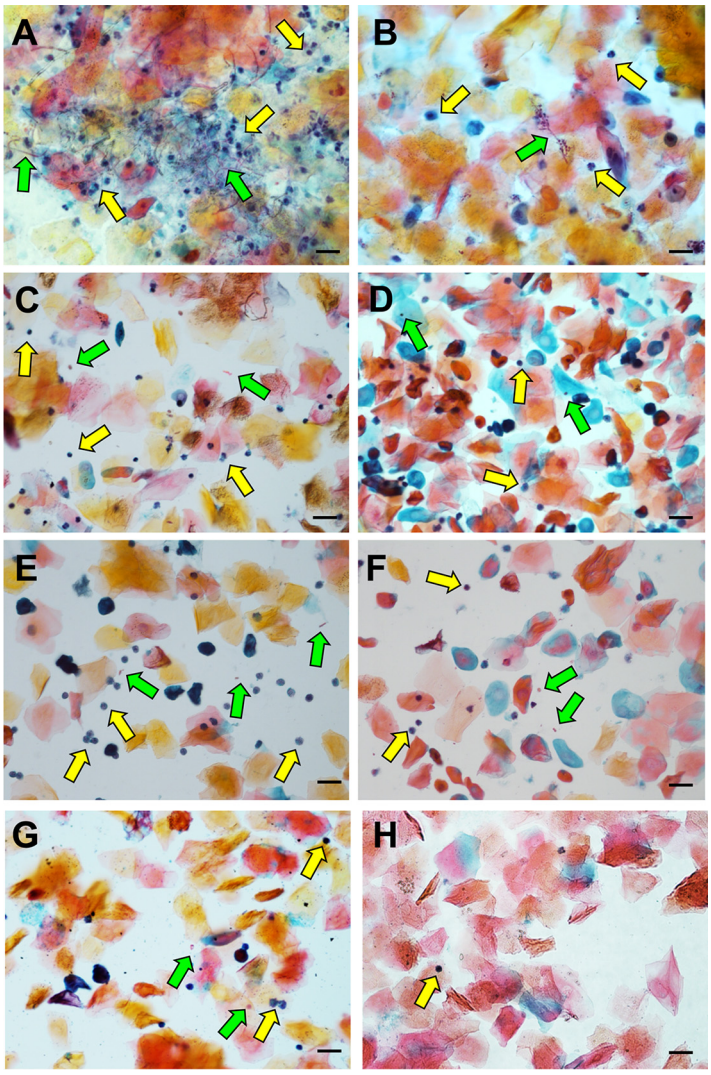

FIG 2 NAC species largely fail to robustly form hyphae or recruit neutrophils *in vivo*. Vaginal lavage fluids (10 μl) from day 3 p.i. were smeared onto glass slides, fixed, and stained by the Papanicolaou technique. Images of five nonadjacent fields were captured by standard light microscopy, and a representative of each is depicted for *C. albicans* (A), *C. dubliniensis* (B), *C. tropicalis* (C), *C. parapsilosis* (D), *C. krusei* (E), *C. glabrata* (F), *C. auris* (G), and mock-challenged mice (H). The green arrows indicate fungi, and the yellow arrows indicate neutrophils. Scale bars, 20 μm.

of each *Candida* species (multiplicity of infection [MOI], 5:1). With the exception of *C. dubliniensis*, the NAC species elicited only negligible IL-1β release during challenge (Fig. 3). Although modest amounts of IL-1β were observed during *C. dubliniensis* challenge, the levels induced by all the NAC species were significantly lower than the levels induced by *C. albicans*. Despite these differences, at least some IL-1β release was observed for all the species tested, and it was largely NLRP3 dependent, as challenge of NLRP3$^{-/-}$ cells led to significantly reduced (or, in some cases, absent) levels of this cytokine. Challenge with lipopolysaccharide (LPS) and ATP (strong activators of the NLRP3 inflammasome) led to NLRP3-dependent IL-1β release equivalent to that observed during *C. albicans* challenge, indicating robustness of the experimental system (Fig. 3). Thus, in comparison to *C. albicans*, these representative NAC species are impaired in their relative capacities to activate the NLRP3 inflammasome.

***ECE1* gene expression is significantly reduced in the NAC species.** We have previously demonstrated that expression of the *ECE1* gene encoding candidalysin is required for robust immunopathology at the vaginal mucosa during *C. albicans* infec-

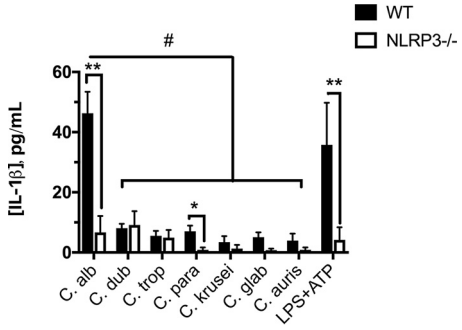

FIG 3 NAC species fail to robustly activate the NLRP3 inflammasome. *C. albicans* and NAC species (*C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *C. auris*) were added to differentiated WT and NLRP3$^{-/-}$ THP-1 cells at an MOI of 5:1 for 4 h. Cells were also challenged with LPS plus ATP as a positive control for inflammasome activation. Cell-free culture supernatants were analyzed for IL-1β release. The solid bars represent WT cells, and the open bars depict NLRP3$^{-/-}$ cells. The numbers of technical replicates ($n = 4$) per experiment were averaged. The data are depicted as the average of 3 independent biological repeats (means plus SEM). Statistical significance was calculated using one-way ANOVA and Dunnett's posttest. Comparison between WT and NLRP3$^{-/-}$ cells, *, $P < 0.05$, and **, $P < 0.01$; comparison between species, #, $P < 0.05$.

tion. It is also highly inducible under conditions that promote the yeast-to-hypha switch (even at time points before hyphae are visually observable) (32). *C. dubliniensis* and *C. tropicalis* are the only *Candida* species with known *ECE1* orthologs, so we used a quantitative real-time PCR (qRT-PCR) approach with species-specific primers (Table 1) to quantify the relative expression of *ECE1* at 4 h after the switch to RPMI medium. Importantly, the primers were designed to yield equivalent PCR efficiencies and were confirmed prior to qRT-PCR analysis (data not shown). As expected, *C. albicans* robustly upregulated *ECE1* expression (~5,000-fold) (Fig. 4A). However, *C. dubliniensis* only modestly expressed *ECE1* (~50-fold), while only exceedingly little induction was detected for *C. tropicalis* (Fig. 4A). In vivo *ECE1* expression was also determined following intravaginal challenge, with *C. albicans* demonstrating robustly elevated levels (Fig. 4B). Consistent with significantly reduced expression levels *in vitro*, quantifiable *ECE1* transcripts could not be reliably detected for *C. dubliniensis* and *C. tropicalis*. Expression levels of *ACT1* for these species were similar to that of *C. albicans*, indicating robustness of the extraction method (data not shown). These results largely correlate with the relative inflammasome activation observed in THP-1 cells and the capacity to switch from yeast to hypha.

DISCUSSION

A plethora of large-scale retrospective and prospective clinical studies examining thousands of isolates have demonstrated that *C. albicans* is clearly the most frequent

TABLE 1 Primer names and sequences used to determine *ACT1* and *ECE1* expression in *C. albicans*, *C. dubliniensis*, and *C. tropicalis*

Primer name[a]	Sequence (5′→3′)
CaACT1qPCR-F	TTGGATTCTGGTGATGGTGTTA
CaACT1qPCR-R	TCAAGTCTCTACCAGCCAAATC
CaECE1qPCR-F	TTGCTAATGCCGTCGTCAGA
CaECE1qPCR-R	GAACGACCATCTCTCTTGGCAT
CdACT1qPCR-F	GGATTCTGGTGATGGTGTTAC
CdACT1qPCR-R	GTAAGTCTCTACCAGCCAAATC
CdECE1qPCR-F	GTTACTAATGCCATCGTCAGA
CdECE1qPCR-R	CAACACCGTCTCTCTTGG
CtACT1qPCR-F	CTTGGATTCTGGTGATGGTGTTA
CtACT1qPCR-R	TCAAGTCTCTACCAGCCAAGTC
CtECE1qPCR-F	GCACTCAAATTCTTGGCTCA
CtECE1qPCR-R	GCTGAAGGTATCAGAATTGGC

[a]Ca, *C. albicans*; Cd, *C. dubliniensis*; Ct, *C. tropicalis*.

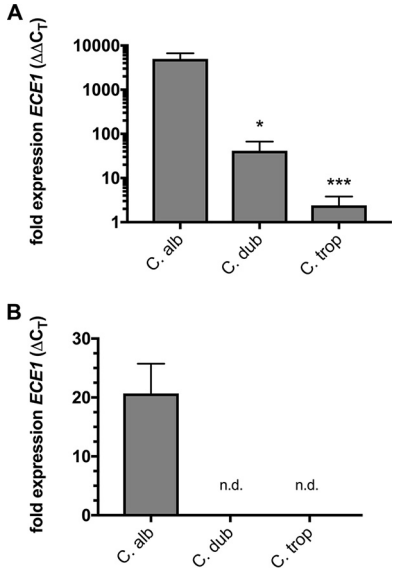

FIG 4 *C. dubliniensis* and *C. tropicalis* fail to strongly upregulate *ECE1* expression *in vitro* or *in vivo*. (A) Overnight YPD cultures of *C. albicans*, *C. dubliniensis*, and *C. tropicalis* were transferred to either fresh YPD or RPMI medium for 4 h. RNA was extracted, normalized, and reverse transcribed, and the expression of *ECE1* and the housekeeping gene *ACT1* was monitored. The normalized fold expression of *ECE1* (mean plus SEM) was calculated from the average independent biological repeats ($n = 3$) using the $\Delta\Delta C_T$ method. Statistical significance was calculated using one-way ANOVA and Dunnett's posttest. *, $P < 0.05$; ***, $P < 0.001$. (B) *C. albicans*, *C. dubliniensis*, and *C. tropicalis* were intravaginally inoculated in estrogen-treated mice as described in the text. At day 3 p.i., the mice underwent two rounds of vaginal lavage with PBS to recover fungal cells, and RNA was immediately prepared as described above. The normalized fold expression of *ECE1* (mean plus SEM) was calculated from the average of independently inoculated animals ($n = 4$) and compared to *ACT1* expression using the ΔC_T method. n.d., not detected.

cause of VVC; commonly reported incidence rates suggest 70 to 90% of the total cases observed per study (18, 20, 23). The gold standard for determining the species of recovered vaginal isolates remains plating on chromogenic agar, which distinguishes *Candida* species based on colony color (33). While distinct color phenotypes do exist for several of the species (including *C. albicans*, *C. tropicalis*, and *C. krusei*), differences between the remaining species can be difficult to distinguish, even for the trained laboratory technician (34). Moreover, *C. dubliniensis* is almost indistinguishable from *C. albicans* using this methodology, possibly leading to low reported rates of *C. dubliniensis* as an etiological agent of VVC (35). That said, molecular diagnostics (e.g., PCR) to distinguish *Candida* species largely report findings similar to those of culture-based identification (12, 24, 36). Thus, imperfect or infrequently used diagnostic tools, self-diagnosis, and availability of over-the-counter treatment options may contribute to the potentially underreported rate of NAC vaginitis.

Interestingly, a number of small-scale (hundreds of isolates) clinical studies have revealed quite a different scenario, in which NAC species make up as much as 50% of the total isolates recovered (13, 15, 37). In some situations, NAC species isolated even outnumber *C. albicans* (17, 38, 39). These somewhat surprising results could be explained as follows. In many cases, the findings arise from assessing symptomatic infection in African or Asian populations, suggesting either ancestral host genetic susceptibility to NAC isolates or geographic dominance of a given species or strain. Also, many of the studies examined patient populations seeking treatment at tertiary-care vaginitis clinics, which are sought only after routine treatment therapy has failed or when chronic recurrence is an issue. Given the association between recurrence and treatment failure with the NAC species, results from these studies may be biased by an already susceptible patient population (22). Nonetheless, our data strongly support the broad clinical finding that *C. albicans* is more adept at causing symptomatic VVC, thus providing a rationale for its increased incidence compared to NAC species.

Our results largely mirror the reduced pathogenicity of the NAC species compared to *C. albicans* reported during infection of oral epithelium (40). Findings by Moyes et al. revealed that of all the pathogenic *Candida* species tested, only *C. albicans* and *C. dubliniensis* were capable of activating mitogen-activated protein kinase (MAPK) signaling in oral epithelial cells—a process strongly correlated with hypha formation (41). However, mucosal damage and downstream cytokine signaling induced by *C. dubliniensis* were significantly impaired in comparison to *C. albicans*, which robustly activated these responses. Furthermore, the remaining fungi tested (*C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, and *Saccharomyces cerevisiae*) failed to approach the magnitude of responses observed during *C. albicans* challenge (40). Similar to these findings, other studies have demonstrated that NAC species inefficiently activate the NLRP3 inflammasome compared to *C. albicans* (Fig. 3), requiring a much higher MOI and/or greatly expanded kinetic time courses (42–44). However, our study represents the first systematic *in vivo* comparison of the pathogenicities of the major candidal causative agents of VVC.

Despite the strong disparity between *C. albicans* and NAC species pathogenicities delineated in this study, we recognize that these findings should be taken with caution. Genome-sequenced reference isolates represent our greatest understanding of an organism's biology and virulence, as significant amounts of published work regarding pathogenicity and fitness exist for these strains. Thus, in this study, we chose to use common reference isolates (or those with whole-genome sequences available) to comparatively assess vaginopathogenicity. However, these data are representative of a single isolate of each species, and the phenotypes should not be broadly conferred across all clinical isolates. For example, Asmundsdottir and colleagues demonstrated that murine systemic infection with different *C. dubliniensis* clinical isolates ($n = 9$) led to lethal outcomes resembling that of *C. albicans* infection in 30% of the cases (45). The remaining isolates were largely avirulent, highlighting dramatic interstrain variability. A similar outcome may likely be true for the other species and/or mucosal disease, including that caused by *C. albicans*. Differences in biofilm formation and hemolysin, protease, or phospholipase secretion likely impact strain-to-strain pathogenicities (46–48).

This possibility raises some intriguing questions. Given that vaginal colonization is not dependent on invasiveness (as evidenced by lack of robust lactate dehydrogenase [LDH] release elicited by *C. dubliniensis*), why has *C. albicans* evolved to be so highly vaginopathogenic? For a commensal organism that inhabits humans as its primary niche, alarming the immune system seems a counterintuitive long-term colonization strategy. However, several plausible hypotheses may explain this. Humans, like mice, are more susceptible to VVC during periods of estrogenic activity (49). Indeed, estrogen induces keratinization of the vaginal epithelium, causing the cornified cells to slough away from the vaginal wall (50). Perhaps *C. albicans* robustly forms hyphae under these conditions to remain anchored to the mucosa to prevent clearance. This hypothesis may be supported by colonization data (Fig. 1), in which species incapable of forming hyphae were largely cleared or exhibited erratic colonization at day 7. However, given that *C. albicans* can covalently bind to epithelial cells (e.g., via Hwp1p-mediated interactions), this hypothesis may provide a less feasible explanation (51). Estrogen also significantly increases the glycogen content of the vaginal epithelium. Dennerstein and Ellis tested a panel of *Candida* species for the capacity to catabolize glycogen *in vitro*. Interestingly, only *C. albicans* was able to utilize this complex carbohydrate source to support growth (52). Thus, it is possible that *C. albicans* has a metabolic advantage at the vaginal mucosa compared to the NAC species. It would be intriguing to investigate genetic disruption of this pathway and its effect on *C. albicans* vaginopathogenesis. Also, fine-tuning of *ECE1* expression or evolved allelic diversity among *Candida* species may help explain their relative pathogenicities (Fig. 4). Lastly, it is possible that *C. albicans* is better equipped to deal with stressors and host defense mechanisms encountered in the vaginal environment (53). Whatever the explanation, it is clear that the capacity to form hyphae and cause damage seemingly confers selective fitness at the vaginal mucosa, given the high isolation rate of *C. albicans* during VVC.

Despite the relative frequency with which they are isolated, the role of NAC species

during VVC remains highly contested. Some clinicians feel that NAC species are simply colonizers that are present during an idiopathic bout of vaginal symptoms (11, 14). In support of this, a clinical study by Dennerstein et al. demonstrated that of 44 women presenting with vaginitis-like symptoms and harboring a NAC yeast, 86% reported spontaneous improvement without requiring antifungal intervention (30). On the other hand, the relatively large number (~5% to 10%) of VVC cases universally caused by *C. glabrata* cannot be denied, suggesting that NAC species do actively contribute to symptomatic VVC (20, 23, 24, 28). *C. glabrata* remains a formidable clinical challenge, as these infections are largely unresponsive to standard fluconazole therapy, given the intrinsic resistance of *C. glabrata* to the azole class (54). Often, prolonged treatment regimens (weeks to months) with alternative topical azole (e.g., miconazole and terconazole) or antifungal (e.g., flucytosine and amphotericin B) drugs are required, in conjunction with boric acid vaginal suppositories (55, 56). However, rates of recurrence and relapse remain unacceptably high.

C. glabrata is unable to form hyphae, has no candidalysin ortholog, and exhibits virtually no immunopathogenicity in the murine model of VVC, yet it clearly does so in women. How is this so? The simplest explanation is that clear differences in human immunity result in exacerbated symptomatology compared to murine challenge. It is also possible that specific isolates of *C. glabrata* are vaginopathogenic while others only asymptomatically colonize. Although much less common than *C. glabrata*, the highly antifungal-resistant emerging pathogen *C. auris* has also been implicated in causing VVC (57). In other cases, even the benign food grade yeast *S. cerevisiae* has been isolated as a causative agent of vaginitis (58). Like *C. glabrata*, these species are restricted to growth in the yeast form. Thus, it is possible that mechanisms unrelated to the fungal pathogen drive vaginal symptoms (e.g., itching and burning) in a subset of women. Perhaps allergic or hypersensitivity responses mediated by population level immunogenetics contribute to heightened responses to fungal antigens or virulence determinants. Given that VVC is largely diagnosed based on symptoms (even in the absence of microbiological evidence), it may be better regarded as a syndrome, in which multifactorial root causes result in similar clinical presentations. This classification would help unify pathogenicity mechanisms for which there is solid foundational biological evidence with otherwise inexplicable clinical findings.

Overall, our results demonstrate that, with the exception of *C. dubliniensis*, the representative NAC species are incapable of forming hyphae in the murine vagina and consequently do not elicit robust immunopathology characteristic of *C. albicans*-mediated disease. Consistent with these observations, these NAC species also fail to robustly activate the NLRP3 inflammasome, contributing to reduced immunopathogenicity. These findings provide a supportive rationale for *C. albicans* being the primary causative agent of VVC.

MATERIALS AND METHODS

Ethics statement. The animals used in this study were housed in AAALAC-approved facilities located in the Regional Biocontainment Laboratory (RBL) at the University of Tennessee Health Sciences Center (UTHSC). The UTHSC Animal Care and Use Committee approved all animals and protocols. The mice were given standard rodent chow and water *ad libitum*. The mice were monitored for signs of distress, including noticeable weight loss and lethargy.

Microorganism growth. When possible, genome-sequenced reference isolates were used. *C. albicans* SC5314, *C. dubliniensis* CD36, *C. tropicalis* MYA3404, *C. parapsilosis* CDC317, *C. krusei* 81-B-5 (59), *C. glabrata* CBS138, and *C. auris* [429]0382 were maintained as glycerol stocks stored at −80°C. A small amount of stock was spread onto yeast extract-peptone-dextrose (YPD) agar and incubated at 30°C for 48 h to obtain isolated colonies. A single colony was transferred to 10 ml of YPD and incubated at 30°C with shaking at 200 rpm for 18 h prior to vaginal or THP-1 challenge.

Murine model of vulvovaginal candidiasis. The well-established murine model of vulvovaginal candidiasis was performed as described previously (60, 61). Briefly, female 6- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories and housed in isolator cages mounted on ventilated racks. The mice were administered 0.1 mg of estrogen (β-estradiol 17-valerate; Sigma) dissolved in 0.1 ml of sesame oil subcutaneously 72 h prior to intravaginal inoculation with *Candida* species. Stationary-phase cultures of *Candida* isolates were prepared as described above. Cell suspensions were counted and adjusted to 5×10^8 CFU/ml in sterile endotoxin-free phosphate-buffered saline (PBS). The estrogen-treated mice were intravaginally inoculated with 10 μl of the standardized blastoconidial cell suspension,

generating an inoculum size of 5×10^6 blastoconidia. The mice underwent vaginal lavage with 100 μl of PBS at days 3 and 7 p.i. The resultant lavage fluids were briefly centrifuged (2,500 rpm; 1 min) to remove debris and spiked with 1 μl of 100× EDTA-free protease inhibitors (Complete; Roche) and kept on ice or stored at −80°C until they were processed for immunopathological markers. All animal experiments (n = 4 per group) were conducted in duplicate, and the resulting data were combined.

Assessment of fungal burden and vaginitis immunopathology. Immunopathological markers were assessed as described previously (62). Lavage fluid was serially diluted 10-fold by the drop plate method and plated onto YPD agar containing 50 μg/ml chloramphenicol. The plates were incubated for 24 h at 37°C, colonies were enumerated, and numbers of CFU/ml were reported as the median. Lavage fluid (10 μl) was smeared onto glass slides, fixed with CytoFix spray, and stained by the Papanicolaou technique to assess PMN recruitment (small blue cells with multilobed nuclei). PMNs were counted in 5 nonadjacent fields by standard light microscopy, using a 40× objective, and values were reported as means plus standard errors of the mean (SEM). Murine IL-1β was assessed in clarified, diluted vaginal lavage fluid using a murine Ready-Set-Go enzyme-linked immunosorbent assay (ELISA) kit (eBioscience) according to the manufacturer's protocol, and the results were reported as means plus SEM. LDH activity was measured in diluted lavage fluid using the commercially available CytoTox 96 nonradioactive cytotoxicity assay (Promega), and the results were reported as means plus SEM.

Hyphal growth assay. *Candida* species were grown overnight in YPD, washed in PBS as described above, diluted to 5×10^5 cells/ml in phenol red-free RPMI 1640 containing 25 mM HEPES (Corning), and shaken in a 37°C incubator. Aliquots were removed at several time points, and wet mounts were prepared and imaged by standard light microscopy.

Cultivation of cell lines. WT (THP1 null; InvivoGen) and NLRP3$^{-/-}$ (THP1-defNLRP3; InvivoGen) THP-1 cells were cultured according to the manufacturer's protocol in RPMI 1640 with 25 mM HEPES supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml Pen-Strep, and normocin. THP-1 cells were counted on a Countess II FL (Life Technologies) and frozen as aliquots of $\sim 5 \times 10^6$ cells in liquid nitrogen.

Macrophage challenge assay. Upon recovery from cryopreservation, cells were incubated for 3 days at 37°C, 5% CO_2, and 90% humidity in a T25 flask in RPMI 1640 medium (10% heat-inactivated FBS, 100 U/ml Pen-Strep). After 3 days, THP-1 cells were counted on the Countess II, assessed for high viability by exclusionary trypan blue staining, and diluted to 5.56×10^5 cells/ml in RPMI 1640 (25 mM HEPES, 10% heat-inactivated FBS, 100 U/ml Pen-Strep), and 180-μl aliquots were seeded at a final density of 1×10^5 cells/well in 96-well polystyrene plates. Phorbol 12-myristate 13-acetate (PMA) (InvivoGen) was added at a final concentration of 100 nM to differentiate cells to a macrophage phenotype, and the cells were incubated at 37°C with 5% CO_2 for 24 h. Following incubation, the spent culture medium was replaced with 180 μl fresh phenol red-free RPMI 1640 containing 25 mM HEPES. Overnight cultures of *Candida* species were prepared as described above, washed 3 times in sterile PBS, and adjusted to 2.5×10^7 CFU/ml, and 20 μl of the suspension was added to wells containing THP-1 cells, generating a 5:1 MOI. Mock-infected controls using medium alone were also included. A positive control for inflammasome activation was also prepared by challenging cells with 1 μg/ml lipopolysaccharide (*Escherichia coli* 0111:B4; InvivoGen) for an equivalent time, followed by addition of 5 mM ATP (InvivoGen) 30 min prior to the endpoint. The cells were incubated for 4 h and gently centrifuged at 200 × g for 2 min to settle the cells/*Candida*, and 100 μl of culture supernatant was transferred to a polystyrene plate containing 100 μl of prediluted 1× ELISA/enzyme-linked immunosorbent spot (ELISPOT) assay buffer (eBioscience) and stored at −20°C. Culture supernatants were assessed for IL-1β using the Human Ready-Set-Go ELISA kit (eBioScience). ELISA optical density values from mock-infected controls were subtracted from those of *Candida*-challenged samples. Experiments were conducted in technical replicates (n = 4) and repeated independently in triplicate. Data are reported as means plus SEM.

Expression of *ECE1* by qRT-PCR. *C. albicans*, *C. dubliniensis*, and *C. tropicalis* were grown overnight in YPD medium at 30°C, washed in PBS, and added to 5 ml of fresh YPD or phenol red-free RPMI 1640 containing 25 mM HEPES and incubated with shaking (200 rpm) at 30°C or 37°C, respectively, for 4 h. RNA was extracted by the acid phenol-chloroform procedure as previously described, followed by precipitation with 3 M sodium acetate-ethanol, washing in 70% ethanol, and air drying (63). The pellets were resuspended in sterile water, and RNA integrity (1 μl) was assessed by morpholinepropanesulfonic acid (MOPS) gel electrophoresis and visualization of intact 18S and 28S bands. RNA concentrations were measured using a NanoDrop spectrophotometer to assess A_{260}/A_{280}. RNA concentrations were equalized among samples, and 200-ng aliquots were treated with RNase-free DNase (Thermo Fisher) according to the manufacturer's instructions. RNA was reverse transcribed using random hexamers and the RevertAid kit (Thermo Fisher) according to the manufacturer's protocol. *Candida* species-specific forward and reverse primers (final concentration, 0.5 μM) for *ECE1* or *ACT1* open reading frames (ORFs) were used in conjunction with 2× Maxima SYBR green mix (Thermo Fisher) according to the manufacturer's instructions to amplify approximately 100-bp fragments from 20 ng of cDNA. Primers were designed to have equivalent melting temperatures. Primer sets were also validated by PCR, amplifying genomic DNA extracted from each species, and gel electrophoresis was used to confirm amplification of target sequences. qRT-PCRs were monitored and analyzed with an Applied Biosystems 7500 platform and software. Expression levels of *ECE1* were compared to those of a reference gene (*ACT1*) and growth in YPD or RPMI medium using the $\Delta\Delta C_T$ method (64). RNA obtained from vaginal lavage fluids of mice 3 days following challenge with *Candida* species was similarly prepared and analyzed for *ECE1* expression, except that levels were reported as the ratio of *ECE1* to *ACT1* using the ΔC_T method.

Imaging. Standard light microscopy images of Papanicolaou-stained vaginal lavage fluids or cells from the hyphal growth assay were captured using a Nikon Ni-U microscope with the NIS Elements

software package. Images were processed (cropped or resized) using Adobe Photoshop CS3, and any adjustments (e.g., brightness or contrast) were applied uniformly across the entire image using ImageJ (NIH). Images of five nonadjacent fields were taken and are representative.

Statistical analyses. All experiments were conducted using groups of mice ($n = 4$) and repeated in duplicate as determined by power analyses. All data were plotted and analyzed for statistical significance using GraphPad Prism software. Data sets were tested for normality using the Shapiro-Wilks test. Data were compared by one-way analysis of variance (ANOVA) and Dunnett's (parametric) or Kruskal-Wallis (nonparametric) posttest. Graphs in figures are annotated to indicate significance levels.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00527-18.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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